

Amendment under 37 C.F.R. § 1.111  
USSN 09/918,508

**REMARKS**

In this Amendment, claims 1-8, 20 and 21 are amended, claims 28 and 29 are added, and claims 9-19 and 22-27 are withdrawn from consideration. Therefore, after entry of this Amendment, which is respectfully requested, claims 1-8, 20, 21, 28, and 29 will be all of the claims pending in the application.

Initially, Applicants note that, while the Examiner indicated in the Office Action that signed and initialed copies of Applicants' PTO Form(s) 1449 were attached to the Office Action, no PTO Forms-1449 were in fact attached. Accordingly, Applicants respectfully request that such be attached to the next correspondence from the patent office. Specifically, Applicants request signed and initialed copies of the PTO Forms-1449 accompanying the Information disclosure Statements of November 5, 2001, August 22, 2002, and December 13, 2002.

Claims 1 and 20 have been amended to recite a step of comparing test samples with control samples. These amendments are supported by the specification at, for example, page 31, first full paragraph, as well as by original claim 20.

Claim 4 has been amended to recite that the lowered histidine kinase activity is due to a defect in a histidine kinase gene. This amendment is supported by the specification at, for example, page 46, line 10, describing the transformation of TM182 (*Sln1*Δ), an *sln1* genetically defective strain.

Claim 8 has been amended to recite a "partially transmembrane region-deleted type cytokinin receptor. This amendment is supported by the specification at, for example, page 22, line 5.

In addition, Applicants have amended claims 1-8, 20 and 21 to more particularly state that which Applicants regard as their invention. These Amendments are not intended to be narrowing, but are merely intended to impart clarity.

Applicants assert that no new matter has been added. Accordingly, Applicants respectfully request that the Amendment be entered into this application.

**I. Objections to the Specification**

(1) At page 2 of the Office Action, the Examiner objects to the disclosure because it contains an embedded hyperlink, and/or other form of browser-executable code, on pages 12 and 22.

Applicants have deleted the browser-executable code as required by the Examiner. Accordingly, Applicants respectfully request that this objection be withdrawn.

(2) At page 2 of the Office Action, the Examiner objects to the Specification because of the use of the following trademarks: Takara Herculanase<sup>TM</sup>, Takara LA taq<sup>TM</sup>, Phytigel<sup>TM</sup>, Oligotex<sup>TM</sup>, and FPLC pure<sup>TM</sup>. The Examiner requires that all trademarks be capitalized wherever they appear and be accompanied by generic terminology.

Applicants have amended the Specification to use all capital letters with trademarks, and have amended the specification to contain generic terminology. As such, Applicants believe that the Specification is in full compliance with the Examiner's requirement. Applicants respectfully request withdrawal of this objection.

## **II. Objections to the Claims**

At page 2 of the Office Action, the Examiner indicates that claim 8 contains a misspelling of the word “cyctokinin.”

Applicants have corrected the misspelling as required by the Examiner, and request withdrawal of this objection.

## **III. Claim Rejections Under 35 U.S.C. § 112, Second Paragraph**

At page 3 of the Office Action, claims 2-4, 8, 20 and 21 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite.

(1) The Examiner states that the phrase “a cell having a function of directly controlling cell growth by intracellular signal transduction” renders claim 2 indefinite, because the cytokinin receptor has this function and not the cell.

Applicants have amended claim 2 to attribute the function of controlling cell growth to intracellular signal transduction from the cytokinin receptor. This amendment is not intended to be narrowing, but is intended to more clearly state the source of control for growth of the transformed cell. Applicants respectfully request withdrawal of this rejection.

(2) The Examiner states that claim 3 is indefinite because, as written, the cell has a lower histidine kinase activity than itself.

Applicants have amended claim 3 to clarify that the host cell has a “lowered intrinsic histidine kinase activity.” This amendment is not intended to be narrowing, but is intended to clarify the property of the host cell as having a lowered intrinsic histidine kinase activity.

Applicants respectfully request withdrawal of this rejection.

(3) The Examiner states that claim 4 is indefinite for the reason that claim 3 is indefinite above, and in addition, the Examiner contends that the term "intrinsic" is itself indefinite.

First, Applicants have amended claim 4 to clarify that the host cell has a "lowered intrinsic histidine kinase activity." Regarding the term "intrinsic," Applicants assert that this term is not indefinite as it is a term of art that refers to the native activity of a cell. Accordingly, Applicants respectfully request withdrawal of this rejection.

(4) The Examiner states that the term "natural form" renders claim 8 indefinite since forms of different structure could occur in nature.

Applicants have amended subpart (d) of claim 8 to recite a "partially transmembrane region-deleted type cytokinin receptor." Applicants further submit that a "partially transmembrane region-deleted type cytokinin receptor" is sufficiently defined in the specification at page 22. This amendment is not intended to be narrowing, but is merely intended to impart clarity. Accordingly, Applicants submit that claim 8 is not indefinite and respectfully request withdrawal of this rejection.

(5) The Examiner states that the term "section" renders claim 20 indefinite and that the phrase "based on the difference obtained by comparison" renders claim 21 indefinite.

Applicants have amended claim 20 to avoid the use of the term "section." This amendment is not intended to be narrowing, but is intended to impart clarity to claim 20. Accordingly, Applicants assert that claim 20 is not indefinite and Applicants respectfully request that this rejection be withdrawn.

Regarding the phrase “based on the difference obtained by comparison,” Applicants have amended claim 20 to more clearly recite a comparing step. This amendment is not intended to be limiting but is merely intended to clarify the claimed subject matter. Accordingly, Applicants respectfully request that this rejection be withdrawn.

#### **IV. Claim Rejections Under 35 U.S.C. § 112, First Paragraph**

(A) At page 4 of the Office Action, the Examiner rejects claims 1-8 and 20-21 under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Specifically, the Examiner contends that claims 1-8 and 20-21, while enabled for methods wherein the cytokinin receptor comprises SEQ ID NO: 2, 4 or 6, are not enabled for methods involving any cytokinin receptor, or a cytokinin receptor wherein the receptor comprises SEQ ID NO: 2, 4, or 6 with deletions, substitutions, or additions of a plurality of amino acids.

Applicants respectfully submit that the specification enables the full scope of the claims. For example, from the last paragraph of page 23 to page 25, line 12, the specification describes the structural characteristics of cytokinin genes as recognized in the art. Thus, Applicants submit that the specification provides adequate guidance to enable a skilled artisan to use any cytokinin gene with the claimed method, and further, the specification provides adequate guidance to enable a skilled artisan to use the claimed method with a cytokinin receptor having additions, deletions or substitutions of one or a plurality of amino acids, since the specification teaches the important structure and identifying characteristics of a cytokinin gene.

Accordingly, Applicants respectfully request reconsideration and withdrawal of this rejection.

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(B) At page 5 of the Office Action, claims 1, 3-8, 20, and 21 are rejected under 35 U.S.C. § 112, first paragraph, because in the Examiner's opinion, the specification is enabling for a method of analyzing agonist activity by measuring cell growth, but does not provide enablement for methods of analyzing agonist activity by measuring intracellular signal transduction. The Examiner contends that the level of the art is such that cytokinin receptors are not sufficiently understood such that one skilled in the art could practice the invention in ways other than measuring cell growth.

Applicants respectfully submit that the Examiner has mischaracterized the state of the art, and further, Applicants submit that the present specification provides sufficient guidance for determining cytokinin activity by measuring intracellular signal transduction from the cytokinin receptor. For example, page 29 of the specification provides guidance in determining intracellular signal transduction by assaying for histidine kinase activity, and one skilled in the art understands that histidine kinase activity can be used as an assay for cytokinin receptor activity. In addition, Estelle, cited by the Examiner as evidence of the state of the art, indicates that the histidine kinase pathway and the G-protein-coupled receptor pathway are well characterized (see abstract). In addition, Applicants submit that Estelle was published three years before the present application's filing date (1998), and as such Estelle is not a good indication of the state of the art as of 2001.

Also, *Nature*, 409, 1060-1063 (2001), provided with attached section 131 Declaration, shows that the histidine kinase pathway is involved in transducing the intracellular signal for cytokinin receptors.

**V. Claim Rejections Under 35 U.S.C. § 103(a)**

At page 6 of the Office Action, claims 1, 2, 6-8, 20 and 21 are rejected under 35 U.S.C. § 103(a) as obvious over Benfey et al. (U.S. Patent Application Publication 2002/0173017), in view of Iwamura et al., J. Medicinal Chem. 26:6 838-44 (1983). Specifically, the Examiner states that Benfey et al. teach: (1) the WOL gene, (2) plant cells transformed with recombinant constructs expressing the WOL gene, (3) identification of ligands to the WOL cytokinin receptor, (4) that WOL is a two-component signal transducer, (5) methods for identifying compounds that modulate the activity of a WOL polypeptide, and (6) that the WOL gene products may be expressed in yeast. The Examiner admits that Benfey et al. do not teach a method for analyzing agonist activity wherein intracellular signal transduction is measured.

The Examiner states that Iwamura et al. teach a method of exposing cells expressing cytokinin receptors to cytokinin receptor agonists and antagonists, and further teach measuring cytokinin receptor activity by measuring cell propagation.

The Examiner states that it would have been obvious to a person of ordinary skill in the art to modify the method as taught by Benfey et al. by measuring changes in signal transduction as taught by Iwamura et al. The Examiner states that the motivation to combine the teachings of Benfey and Iwamura is provided by Iwamura et al., where Iwamura et al. teach measuring the effects of agonists on cytokinin receptors, and the WOL gene as taught by Benfey et al. is a cytokinin receptor.

Applicants submit that the present invention was invented by Applicants before the filing of the Benfey provisional application 60/253,739 filed on November 29, 2000. As established

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by the attached Declaration Under 37 U.S.C. § 1.131<sup>1</sup>, the present inventors are co-authors of the manuscript published as *Nature*, 409, 1060-1063 (2001) (cited in Applicants' Form-1449 accompanying the August 22, 2002 Information Disclosure Statement). As indicated on the published article, the manuscript was received for publication by the journal *Nature* on October 16, 2000 (see page 1063, left column), before the filing date of the Benfey provisional application. Thus, the present invention was invented by Applicants prior to October 16, 2000, and invented prior Benfey's earliest U.S. filing date. As such, Benfey is not prior art to this application.

Applicants further submit that since Iwamura does not teach or suggest the present claims, that the Examiner has not made a *prima facie* case of obviousness. Therefore, Applicants respectfully request withdrawal of this rejection.

## **VI. Conclusion**

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

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<sup>1</sup> An unexecuted copy of the Declaration is attached to this Amendment. An executed copy of the Declaration will be submitted in due course to complete the record.



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The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

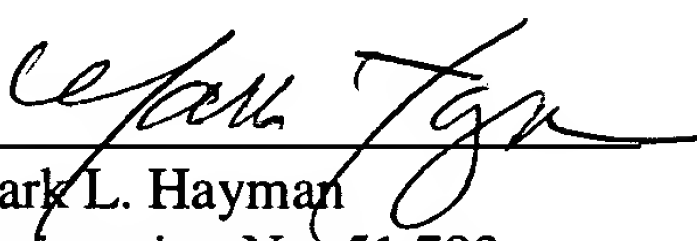
Respectfully submitted,

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WASHINGTON OFFICE

**23373**

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Date: July 6, 2004



**PATENT APPLICATION**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of

Docket No: Q65478

Tatsuo KAKIMOTO, et al.

Appln. No.: 09/918,508

Group Art Unit: 1647

Confirmation No.: 3296

Examiner: Rachel B. Kapust

Filed: August 01, 2001

For: ANALYSIS OF AGONIST-ACTIVITY AND ANTAGONIST-ACTIVITY TO  
CYTOKININ RECEPTOR

**DECLARATION UNDER 37 C.F.R. § 1.131**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

We, TATSUO KAKIMOTO, MASAYUKI HIGUCHI, and TSUTOMU INOUE do hereby  
declare and state:

THAT we are the inventors of the subject matter disclosed and claimed in the above-  
mentioned application;

THAT we are co-authors of *Nature* Vol. 409, 1060-1063 (2001) (a copy of which is  
attached); and

THAT the present invention was invented prior to October 16, 2000, as evidenced by the  
date that the manuscript published as *Nature* Vol. 409, 1060-1063 was received by the Journal  
*Nature* for publication (see page 1063, above references). *Nature* Vol. 409, 1060-1063 shows  
typical working examples of the present invention (see page 1061, right column, lines 15-36, and  
page 1062, Figure 4). *Nature* Vol. 409, 1060-1063 shows *CRE1* gene, which is a typical example  
of a cytokinin receptor gene within scope of the claims. *Nature* Vol. 409, 1060-1063 also shows a  
yeast strain deficient in the *SLN1* gene (*sln1*  $\Delta$  mutant) (page 1061, right column, lines 15-26),

which is a typical example of "a host cell having a lowered intrinsic histidine kinase activity, wherein said intrinsic histidine kinase activity was lowered by the defect in one or more histidine kinase genes". Furthermore, *Nature* Vol. 409, 1060-1063 shows a *sln1*  $\Delta$  mutant carrying p415CYC-CRE1 (page 1061, right column, lines 26-27), which is a typical example of "a cell transformed with DNA comprising a cytokinin receptor gene, wherein the transformed cell expresses said cytokinin receptor from said DNA, and wherein growth of said transformed cell is controlled by intracellular signal transduction from said cytokinin receptor". Moreover, *Nature* Vol. 409, 1060-1063 shows a method for determining a level of intracellular signal transduction by measuring growth of said transformed cell in presence of examinee substance (page 1061, right column, lines 28-29), and determining a second level of intracellular signal transduction by measuring growth of said transformed cell in absence of said examinee substance (page 1061, right column, lines 26-27). *Nature* Vol. 409, 1060-1063 further shows comparing said level and said second level of intracellular signal transduction from said cytokinin receptor (page 1061, right column, lines 26-36, and page 1062, Figure 4). Thus, *Nature* Vol. 409, 1060-1063 shows typical working examples of the claimed method for determining agonist-activity to a cytokinine receptor.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Declaration under 37 C.F.R. § 1.131  
USSN 09/918,508

Date: \_\_\_\_\_

Name: \_\_\_\_\_  
TATSUO KAKIMOTO

Date: \_\_\_\_\_

Name: \_\_\_\_\_  
MASAYUKI HIGUCHI

Date: \_\_\_\_\_

Name: \_\_\_\_\_  
INOUE TSUTOMU

26. Yewdell, J. W., Gerhard, W. & Bachi, T. Monoclonal anti-hemagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid activation of influenza virus A/PR/8/34-mediated hemolysis. *J. Virol.* 48, 239–248 (1983).
27. Porgador, A., Mandelboim, O., Restifo, N. P., Strominger, J. L. Natural killer cell lines kill autologous beta2-microglobulin-deficient melanoma cells: implications for cancer immunotherapy. *Proc. Natl Acad. Sci. USA* 94, 13140–13145 (1997).
28. Brand, C. M. & Skehel, J. J. Crystalline antigen from the influenza virus envelope. *Nature* 238, 145–147 (1972).
29. Irving, B. A. & Weiss, A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell* 64, 891–901 (1991).

#### Acknowledgements

We thank N. P. Restifo for the 1106mel cells; A. Portner from St. Jude Children's Research Hospital for providing the pcn-svhn plasmid; and J. Skehel and R. Gonsalves from National Institute of Medical Research for the purified HA. O.M. is supported by the Israel Science Foundation and The Charles H. Revson Foundation. A.P. is supported by the Israel Science Foundation and by the USA–Israel Binational Science Foundation. O.M. and A.P. are supported by the Israel Cancer Research Foundation.

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## Identification of CRE1 as a cytokinin receptor from *Arabidopsis*

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Cytokinins are a class of plant hormones that are central to the regulation of cell division and differentiation in plants<sup>1,2</sup>. It has been proposed that they are detected by a two-component system, because overexpression of the histidine kinase gene *CKII* induces typical cytokinin responses<sup>3</sup> and genes for a set of response regulators of two-component systems can be induced by cytokinins<sup>4,5</sup>. Two-component systems use a histidine kinase as an environmental sensor and rely on a phosphorelay for signal transduction. They are common in microorganisms, and are also emerging as important signal detection routes in plants<sup>6–9</sup>. Here we report the identification of a cytokinin receptor. We identified *Arabidopsis cre1* (cytokinin response 1) mutants, which exhibited reduced responses to cytokinins. The mutated gene *CRE1* encodes a histidine kinase. *CRE1* expression conferred a cytokinin-dependent growth phenotype on a yeast mutant that lacked the endogenous histidine kinase *SLN1* (ref. 10), providing direct evidence that *CRE1* is a cytokinin receptor. We also provide evidence that cytokinins can activate *CRE1* to initiate phosphorelay signalling.

Generally, cytokinins induce cell division, chloroplast development and formation of shoots (buds)<sup>1</sup>. We screened mutagenized *Arabidopsis* for mutants that were impaired in cytokinin responses, including rapid cell proliferation and shoot formation in tissue culture. We isolated a mutant designated *cytokinin response 1-1* (*cre1-1*). We tested the responses of *cre1-1* to auxin and cytokinin in tissue culture, using naphthalene acetic acid (NAA) as an auxin and kinetin as a cytokinin (Fig. 1). Wild-type explants responded to increasing levels of kinetin with rapid proliferation, greening and formation of shoots (Fig. 1a). By contrast, such cytokinin responses were not evident in *cre1-1* (Fig. 1b). The mutant was also less

responsive to other cytokinins, including *trans*-zeatin, isopentenyladenine, benzyl adenine and the phenylurea-type synthetic cytokinin thidiazuron (see Supplementary Information).

Next we tested the responses of *cre1-1* to various plant hormones in a root elongation assay. External application of cytokinins<sup>11</sup>, ethylene<sup>12</sup>, auxins<sup>13</sup> or abscisic acid<sup>14</sup> inhibits root elongation. The root of the *cre1-1* mutant was less sensitive to benzyl adenine than that of wild-type plants, but it responded normally to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the auxin indole-3-acetic acid (IAA) (Fig. 2a–c). The responses of *cre1-1* to low levels of abscisic acid (ABA) were slightly higher than normal (Fig. 2d). The cytokinin responses of *cre1-1* heterozygotes were intermediate between those of *cre1-1* homozygotes and the wild type (see Supplementary Information).

We mapped the *CRE1* locus to the top of chromosome 2 between the *rga* and *nga145* markers (see Supplementary Information). We searched the genome sequence of *Arabidopsis* between these markers for genes that could code for proteins involved in signal transduction. Among them was the hypothetical gene *At2g01830*, possibly coding for a histidine kinase. The nucleotide sequence of *At2g01830* revealed that this gene was mutated in the *cre1-1* mutant. Hereafter we refer to this gene as *CRE1*. *CRE1* is identical to *WOL*<sup>15</sup> (see below) and *AHK4* (ref. 16). A genomic fragment containing *CRE1* was introduced into *cre1-1* mutant calli. Wild-type calli that had been transformed with the control vector regenerated shoots when cultured in the presence of the

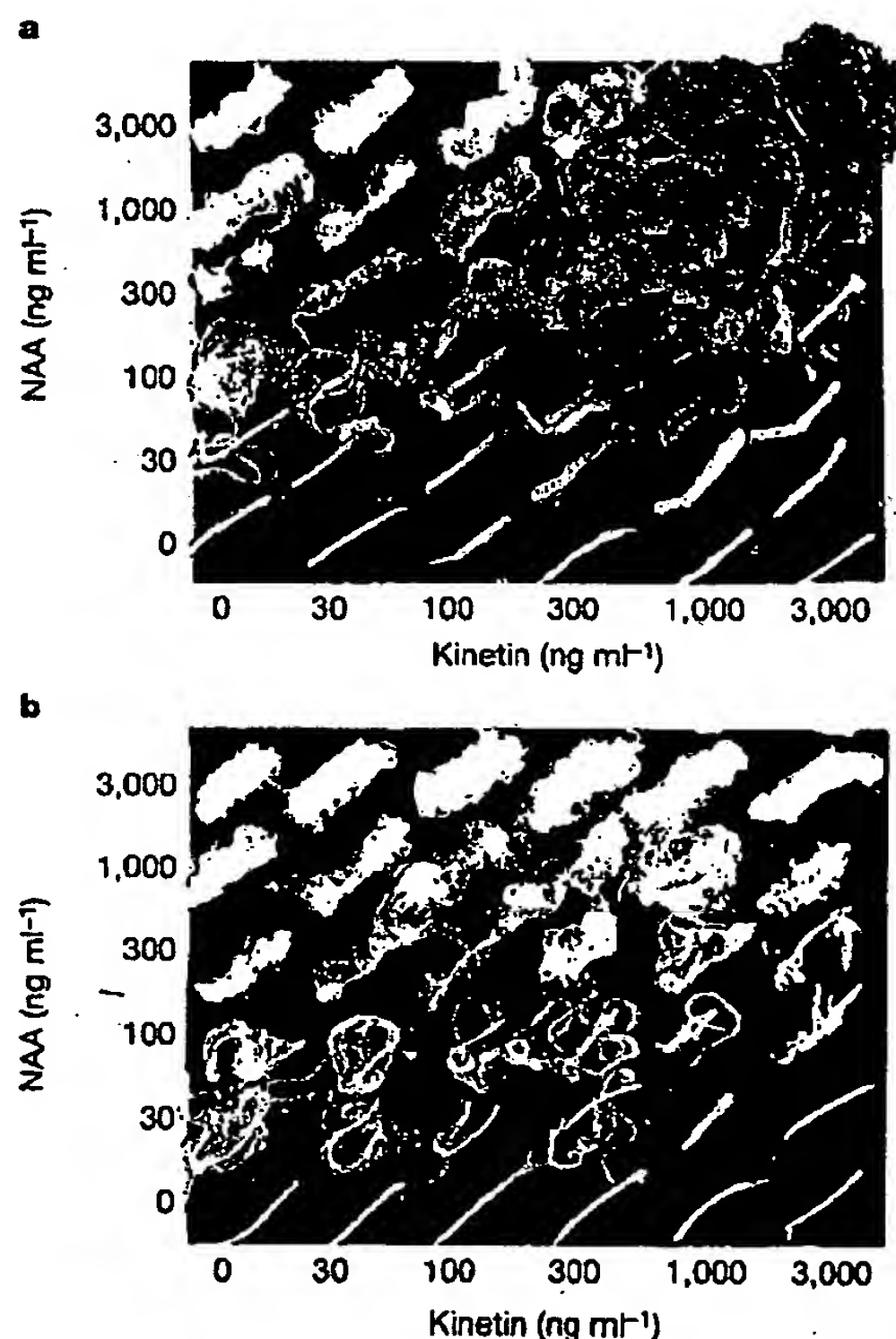


Figure 1 Callus growth of the cytokinin-resistant mutant *cre1-1* in different auxin and cytokinin concentrations. Hypocotyl segments were excised and cultured on media containing different levels of kinetin and NAA. After 21 days in culture, the induced calli were arranged and photographed. Wild-type explants (a) proliferated rapidly, turned green, and produced shoots in the presence of high concentrations of cytokinins. The *cre1-1* explants (b) did not.



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## letters to nature

cytokinin *trans*-zeatin, but *cre1-1* mutant calli transformed with the control vector did not (Fig. 3). However, mutant calli regenerated shoots in the presence of *trans*-zeatin if they had been transformed with pGPTV-KAN-CRE1 (Fig. 3d), indicating that CRE1 complemented the *cre1-1* mutant.

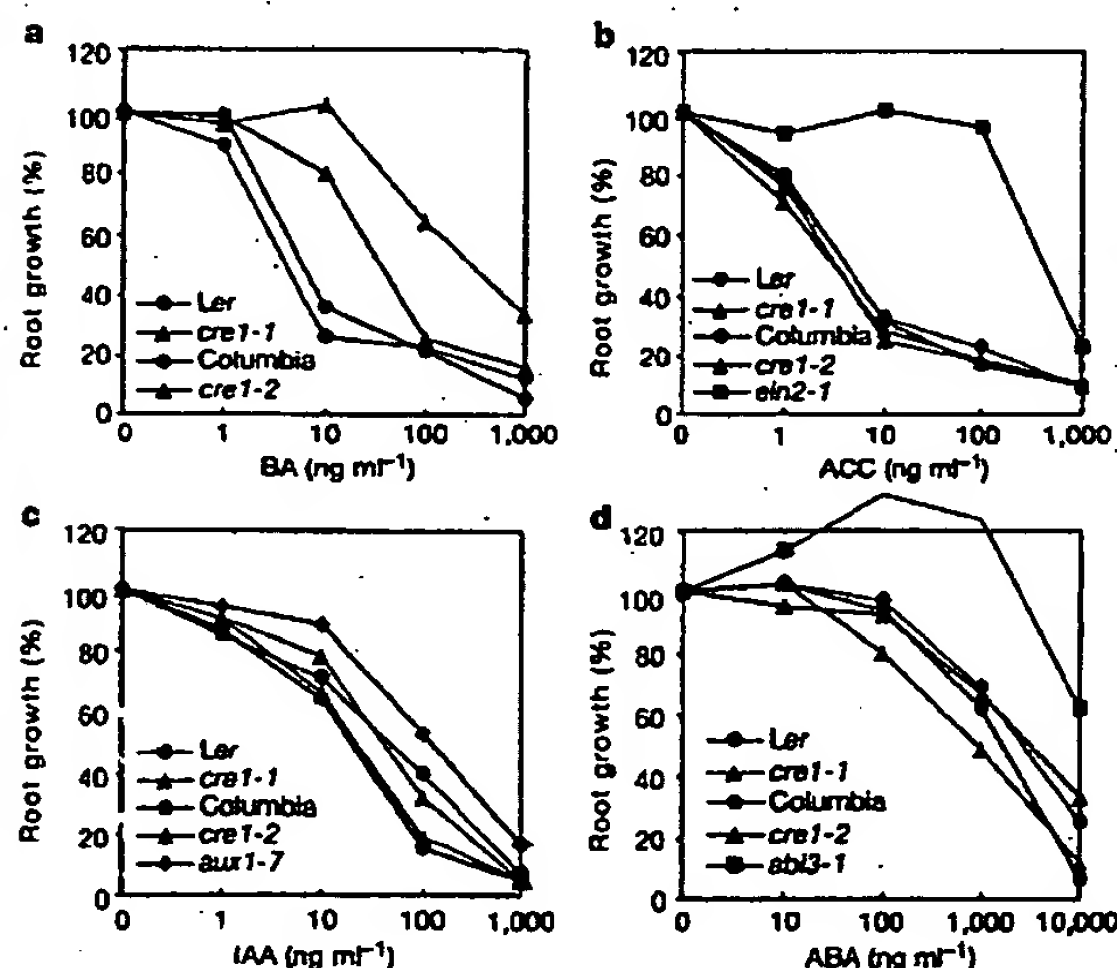
We screened a library of *Arabidopsis* complementary DNA and isolated the corresponding cDNA clones, which were derived from two types of alternately spliced message named CRE1a and CRE1b. The predicted protein for CRE1a consists of 1,057 amino acids and that for CRE1b has 23 extra amino acids at its amino terminus. The CRE1a product was used for further study and for numbering of the amino-acid residues. The carboxy-terminal region of CRE1 carries a histidine kinase domain and a receiver domain. Between these domains, there is another region with weak similarity to receiver domains. According to the PSORT prediction (<http://psort.nibb.ac.jp/form.html>), CRE1 probably localizes to the plasma membrane. The N-terminal region probably consists of an extracellular domain flanked by two transmembrane segments, and the C-terminal region is intracellular. We detected CRE1 message in various tissues (data not shown), but the highest expression was in the root<sup>15</sup>. The *cre1-1* mutation converted Gly 467 in the histidine kinase domain to Asp 467 (see Supplementary Information). *Arabidopsis* has genes for two products, AAF99730 (AHK3 (ref. 16) and BAB09274 (AHK2 (ref. 16)), that share high sequence similarity to CRE1, being 52% and 54% identical, respectively, over their entire proteins, and 61% and 60% identical, respectively, over their extracellular domains. CKII was less similar to CRE1 than these proteins (see Supplementary Information).

We also isolated an *Arabidopsis* line, *cre1-2*, with a T-DNA (see Methods) insertion in the CRE1 gene. The integration occurred in the place of nine base pairs of CRE1 between nucleotide positions +75 and +84 relative to the inferred translation initiation site (see

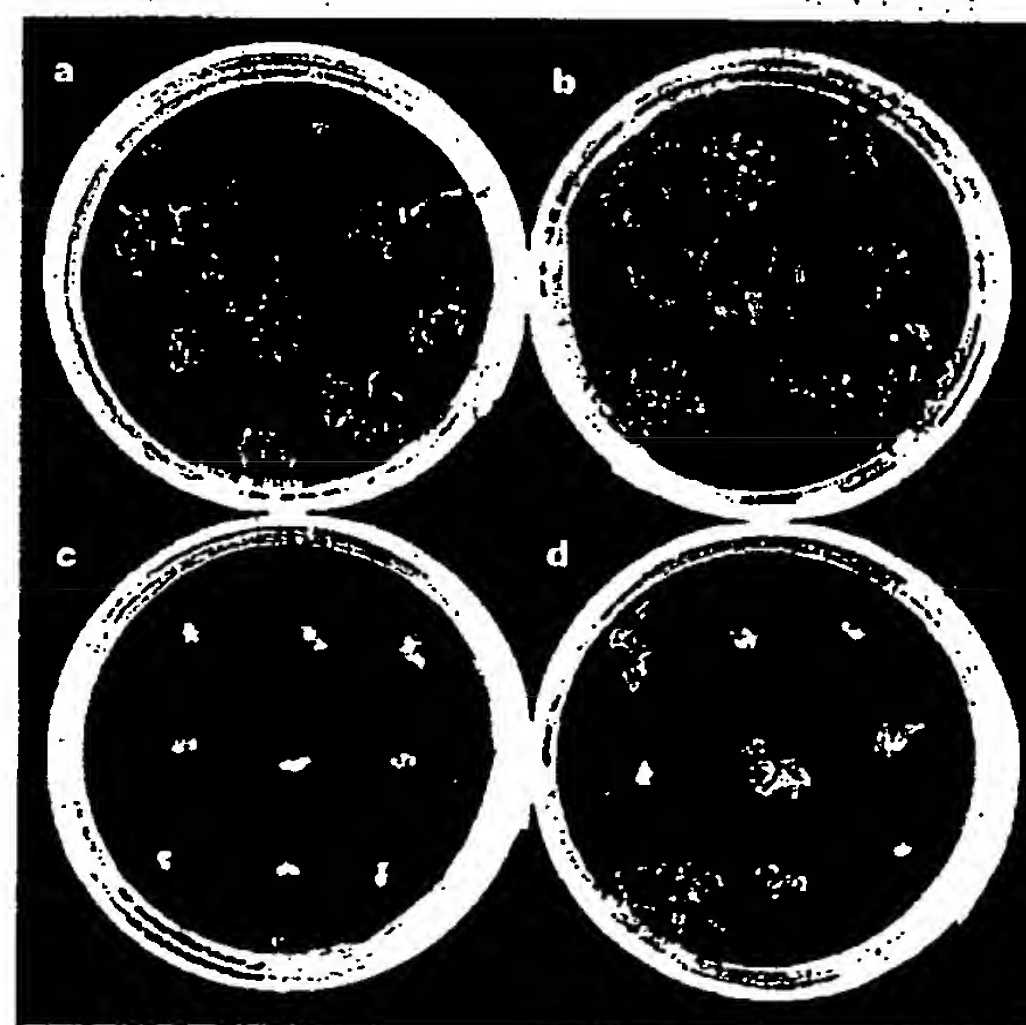
Supplementary Information). The *cre1-2* line, which was homozygous for the T-DNA insertion, was also resistant specifically to cytokinins in the root elongation assay (Fig. 2) and in the callus growth and shoot formation assays (data not shown). The *cre1-2* mutant was complemented by introduction of the CRE1 gene (see Supplementary Information). The presence of the mutation in the CRE1 gene in either of the *cre1-1* or *cre1-2* mutants, and complementation of *cre1-1* and *cre1-2* by CRE1, are definitive evidence that mutations in CRE1 cause the cytokinin-insensitive phenotype of the *cre1* mutants. The *cre1* mutants were allelic to the *wol* mutant (that is, the *cre1* and *wol* mutants bore mutations in the same gene), which is impaired in cell division and proper formation of vascular tissue of the root<sup>15</sup>. The xylem organization of *cre1-1* was also altered (data not shown).

To explore the function of the CRE1 gene, we expressed CRE1 (Fig. 4) in a yeast strain deficient in the SLN1 gene, which encodes an osmosensing histidine kinase<sup>10</sup>. At normal osmolarity, SLN1 autophosphorylates the conserved histidine residue. The phosphoryl group is then transferred to the conserved aspartate residue in the receiver domain of the same protein, then to the phosphotransfer mediator YPD1, and finally to the SSK1 response regulator. This in turn inhibits the ability of SSK1 to activate the downstream mitogen-activated protein (MAP) kinase pathway<sup>17</sup>. The *sln1Δ* mutant is lethal because the downstream SSK1 is always dephosphorylated, which overactivates the downstream MAPK pathway<sup>10,17</sup>. The *sln1Δ* mutant carrying p415CYC-CRE1, which would express the CRE1 gene, was still lethal without cytokinins. However, surprisingly, it grew at a normal rate if *trans*-zeatin, a native cytokinin, was included in the medium. It is noteworthy that the active cytokinin *trans*-zeatin<sup>18</sup> was effective in this yeast system, but the much less active cytokinin *cis*-zeatin<sup>18</sup> was ineffective (Fig. 4a). Other active cytokinins—*isopentenyladenine*, *benzyladenine* and *thidiazuron*—were also effective. The plant hormones IAA, gibberellin A<sub>3</sub> and abscisic acid had no effect. Expression of CRE1b, another form of alternatively spliced product, in the *sln1Δ* mutant gave the same results (data not shown).

p415CYC-CRE1 did not suppress the lethality of the *ypd1Δ* mutant on plates either with or without cytokinins, indicating that

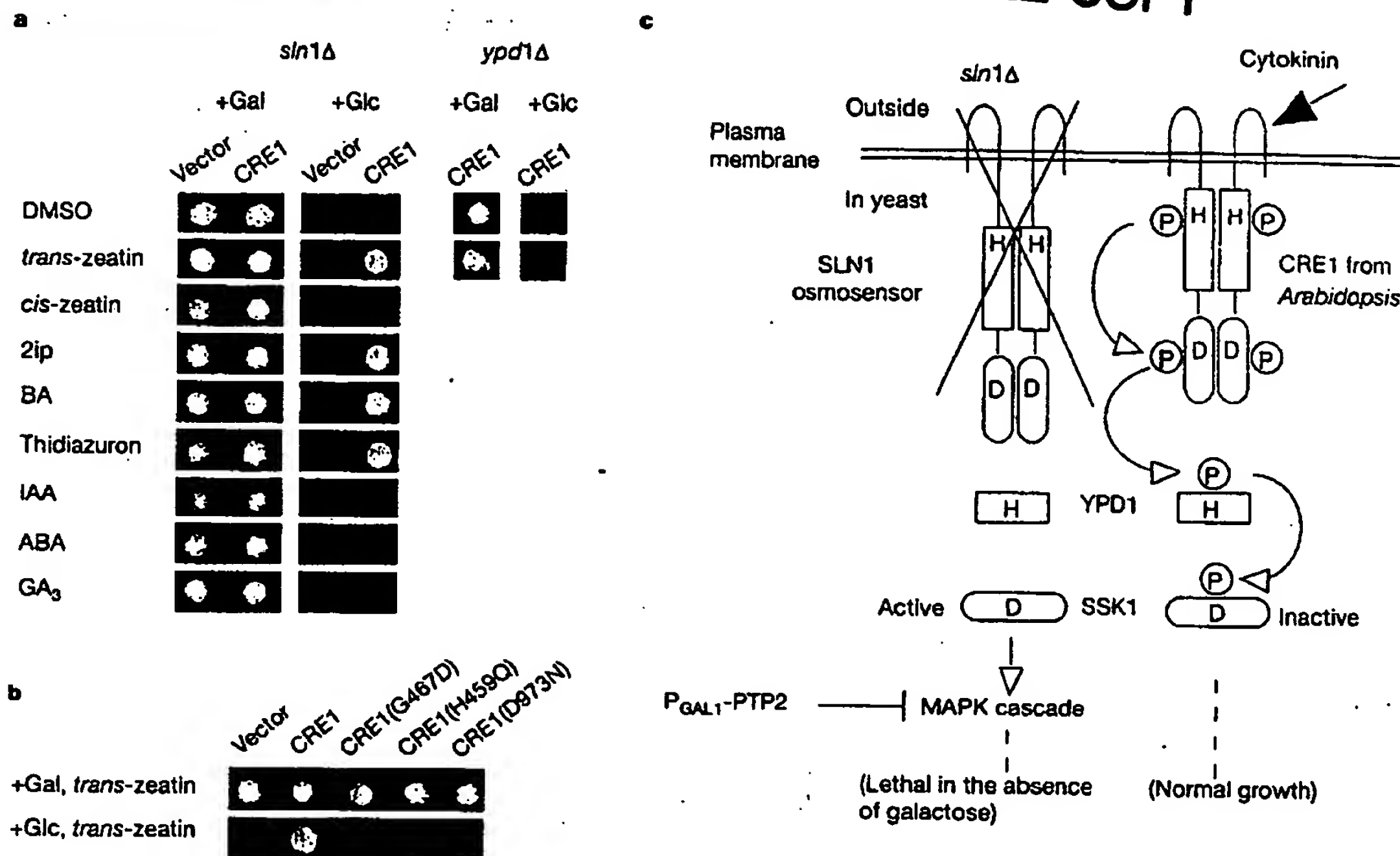


**Figure 2** Root growth of the *cre1-1*, *cre1-2* and hormone-related mutants in the presence of various plant hormones. Seeds were sown on GM plates<sup>22</sup> containing BA (a), ACC (b) or IAA (c). After chilling for three days, plates were incubated at 23 °C for eight days and the root lengths were measured. To determine ABA response, two-day-old seedlings germinated on GM were moved onto plates containing different concentrations of ABA and cultured for six days (d). Root growth was expressed relative to the mean root elongation of the same genotype on the medium without plant hormones. Each value represents the mean of at least 11 plants. *cre1-1* and *abi3-1* have Ler genetic background (red symbols); *cre1-2*, *ein2-1* and *aux1-7* have Columbia genetic background (black symbols). The root length of each genotype in the absence of plant hormones shown in a–d is given in the Supplementary Information.



**Figure 3** Complementation of *cre1-1* by CRE1. Wild-type calli (a, b) or *cre1-1* calli (c, d) were transformed with pGPTV-KAN (a, c) or pGPTV-KAN-CRE1 (b, d) and cultured for 19 days with 0.5 μg ml<sup>-1</sup> *trans*-zeatin and 0.3 μg ml<sup>-1</sup> indole butyric acid (an auxin). Shoots regenerated from different calli are independent transformants, and those on the same callus may or may not be independent.

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**Figure 4** Cytokinin-dependent growth phenotype of yeast in which *SLN1* had been replaced with *CRE1*. **a**, *sln1Δ* and *ypd1Δ* strains were transformed with the vector p415CYC (vector) or p415CYC-*CRE1* (*CRE1*). Suspensions of transformants were spotted onto a plate containing a plant hormone as indicated, and galactose (+Gal) or glucose (+Glc). **b**, Effect of the mutation that was present in the *Arabidopsis cre1-1* mutant (G467D), or of the mutation that changed the conserved His 459 or Asp 973

phosphorylation sites (H459Q or D973N respectively). **c**, The presumed events in yeast. *CRE1* suppresses the lethality of *sln1Δ* but not *ypd1Δ* in the presence of cytokinins. The *sln1Δ* mutant is lethal because the dephosphorylated SSK1 constitutively activates the MAPK pathway. Cytokinins probably activate the histidine kinase activity of the *CRE1* protein to initiate the phosphorelay, whereby the phosphoryl group is transferred from the activated *CRE1* to YPD1, then to SSK1, suppressing the lethality of *sln1Δ*.

signal transduction from *CRE1* is mediated by YPD1 in yeast (Fig. 4c). We next introduced the mutation that was present in the *Arabidopsis cre1-1* mutant into p415CYC-*CRE1*. The resulting plasmid, p415CYC-*CRE1*(G467D), could not suppress the lethality of *sln1Δ* either with or without *trans*-zeatin, indicating that the *CRE1* gene of the *Arabidopsis cre1-1* mutant was nonfunctional (Fig. 4b). Mutations at either the conserved His 459 or Asp 973 of the phosphorylation site in the histidine kinase or the receiver domains, respectively, also destroyed the ability of *CRE1* to suppress the lethality of the *sln1Δ* mutant (Fig. 4b). Therefore, cytokinins probably activate the histidine kinase activity of *CRE1*, and the signal is probably transmitted through YPD1 to SSK1. *Arabidopsis* also has phosphotransfer mediators<sup>20,21</sup>, which resemble YPD1, and response regulators<sup>45,7</sup>. Therefore, in plants, cytokinins probably activate *CRE1* and possibly its homologues, which in turn initiate the phosphorelay signalling that governs cytokinin responses.

In *Arabidopsis*, ethylene receptors<sup>8,9</sup> and possibly osmosensors<sup>19</sup> are histidine kinases. CK11 histidine kinase has been implicated in the detection or signal transduction (or both) of cytokinins<sup>3</sup>, but its function has yet to be clarified. We have provided evidence that the *CRE1* histidine kinase is a cytokinin receptor: mutations in the *CRE1* gene caused a cytokinin-insensitive phenotype in *Arabidopsis*, and expression of *CRE1* conferred a cytokinin-responsive phenotype on yeast. The *cre1* and *wol* mutants were impaired in the cell division and differentiation that is essential for proper formation of the root vascular tissue<sup>19</sup>. This observation, coupled with our data, underlines the importance of cytokinin signalling in this process. The *CRE1* homologues AAF99730 and BAB09274 may

also function as cytokinin receptors, which may explain why defects in *CRE1* did not cause more diverse phenotypes related to cytokinin functions.

## Methods

### Screening for mutants impaired in cytokinin responses

Seeds of *A. thaliana* var. *Ler* were mutagenized with ethyl methanesulphonate, and seeds obtained after self-pollination (M2 seeds) were used<sup>22</sup>. Hypocotyl segments of M2 seedlings were aseptically excised and cultured on GM medium<sup>22</sup> supplemented with 100 ng ml<sup>-1</sup> of 2,4-dichlorophenoxyacetic acid (2,4-D), 100 ng ml<sup>-1</sup> of kinetin, and vitamins (100, 10, 1, 1 and 1 μg ml<sup>-1</sup>, respectively, of inositol, thiamine, nicotinic acid, pyridoxine HCl and biotin). Kinetin of this concentration is sufficient to induce cytokinin responses in wild-type explants, including induction of calli with rapid growth and greening without forming root primordia. The top portion of the plant, corresponding to each hypocotyl segment, was grown on GM medium. Calli with a reduced green colour with many root primordia, which usually occur under low levels of kinetin, were chosen as mutant candidates, and their seeds were obtained by growing the corresponding top portions. From about 19,000 M2 seedlings, one line was confirmed for the heritability of the callus phenotypes. Root growth was measured on GM medium supplemented with a plant hormone as described in Fig. 2.

### Transformation of calli

A genomic region encompassing the *CRE1* gene was amplified by polymerase chain reaction (PCR) from genomic DNA of *Ler* using primers 5'-AGCACAATGTGAGTTT-CACTGGCCTC-3' and 5'-AGCTCAAGTCGTCGACTGAGCTATAG-3'. The amplified fragment was digested with *Sal*I and cloned into the pGPTV-KAN<sup>23</sup> vector between the *Sma*I and *Sal*I sites. The sequence of the resultant construct, pGPTV-KAN-*CRE1*, was confirmed and was transformed into *Arabidopsis* calli by the *Agrobacterium*-mediated method, as described<sup>24</sup>, except that hormone concentrations of CIM medium<sup>24</sup> were changed to 0.5 μg ml<sup>-1</sup> 2,4-D and 0.5 μg ml<sup>-1</sup> kinetin. The transformed calli were cultured on GM medium supplemented with 50 μg ml<sup>-1</sup> kanamycin sulphate, 100 μg ml<sup>-1</sup> cefotaxime, 100 μg ml<sup>-1</sup> vancomycin, 0.3 μg ml<sup>-1</sup> indole butyric acid and 0.5 μg ml<sup>-1</sup> *trans*-zeatin, and the same vitamins as were used in the medium for mutant screening. Other culture conditions were as described<sup>1</sup>.



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## Screening for T-DNA insertion lines

We used the T-DNA insertion-line screening system organized at the Kazusa DNA Research Institute. The principles of the screening method were as described<sup>25</sup>. Gene-specific primers were 5'-ATATGCGATAGCGACTCTCGTACAA-3' and 5'-AAC-CAAAATGCATATCAATCAGCAG-3'. T-DNA (pPCVIGEN4HPT)<sup>26</sup> specific primers were 5'-ATAACGCTGCGGACATCTAC-3' and 5'-ATCTAGGCTTTGATAGTCAC-3'. We used four combinations of primer sets, each consisting of a gene specific primer and a T-DNA-specific primer. The position of the T-DNA insert was determined by sequencing the PCR products carrying the T-DNA-genome junctions.

## Yeast experiments

The entire coding region of the CRE1a cDNA was PCR-amplified and cloned into the yeast expression vector p415CYC<sup>27</sup> under the CYC1 promoter at the *Sma*I site, generating p415CYC-CRE1. We used the QuickChange site-directed mutagenesis kit (Stratagene) to generate p415CYC-CRE1(G467D), p415CYC-CRE1(H459Q) and p415CYC-CRE1(D973N). After sequence confirmation, plasmids were introduced into *sln1Δ* (strain TM182<sup>28</sup>) or *ypd1Δ* (strain SW100<sup>17</sup>). Suspensions of transformants were spotted (about 800 cells per spot) onto drop-out media with 10 μM plant hormones as indicated in Fig. 4, with either 2% glucose or 2% galactose.

Received 16 October; accepted 8 December 2000.

1. Skoog, E. & Miller, C. O. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Ser. Exp. Biol.* 11, 118–131 (1957).
2. Mok, M. C. in *Cytokinins* (eds Mok, D. W. S. & Mok, M. C.) 155–166 (CRC, Boca Raton, 1994).
3. Kakimoto, T. CK11, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274, 982–985 (1996).
4. Sakakibara, H. et al. A response-regulator homologue possibly involved in nitrogen signal transduction mediated by cytokinin in maize. *Plant J.* 14, 337–344 (1998).
5. Brandstätter, I. & Kieber, J. I. Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in *Arabidopsis*. *Plant Cell* 10, 1009–1019 (1998).
6. Mizuno, T. His-Asp phosphotransfer signal transduction. *J. Biochem.* 123, 555–563 (1998).
7. D'Agostine, I. B. & Kieber, J. I. Phosphorelay signal transduction: the emerging family of plant response regulators. *Trends Biochem. Sci.* 24, 452–456 (1999).
8. Chang, C., Kwok, S. F., Bleecker, A. B. & Meyerowitz, E. M. *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. *Science* 262, 539–544 (1993).
9. Schaller, G. E. & Bleecker, A. B. Ethylene-binding sites generated in yeast expressing the *Arabidopsis* *ETR1* gene. *Science* 270, 1809–1811 (1995).
10. Maeda, T., Wurgler-Murphy, S. M. & Saito, H. A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* 391, 242–245 (1994).
11. Cary, A. L., Liu, W. & Howell, S. H. Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. *Plant Physiol.* 107, 1075–1082 (1995).
12. Bleecker, A. B., Estelle, M. A. & Somerville, C. Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* 241, 1086–1089 (1988).
13. Lincoln, C., Britton, J. H. & Estelle, M. Growth and development of the *axr1* mutants of *Arabidopsis*. *Plant Cell* 2, 1071–1080 (1990).
14. Kormann, M., Reuling, G. & Karssen, C. M. The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiol. Plantarum* 61, 377–383 (1984).
15. Alahiane, A. P. et al. A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. *Genes Dev.* 14, 2938–2943 (2000).
16. Ueguchi, C., Kizumai, H., Suzuki, T. & Mizuno, T. Novel family of sensor histidine kinase genes in *Arabidopsis thaliana*. *Plant Cell Physiol.* (in the press).
17. Posas, F. et al. Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. *Cell* 66, 865–875 (1996).
18. Schnitz, R. V., Skoog, E., Playin, A. J. & Leonard, N. J. Cytokinins: Synthesis and biological activity of geometric and position isomers of zeatin. *Plant Physiol.* 50, 702–705 (1972).
19. Urao, T. et al. A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *Plant Cell* 11, 1743–1754 (1999).
20. Miyata, S., Urao, T., Yamaguchi-Shinozaki, K. & Shinozaki, K. Characterization of genes for two-component phosphorelay mediators with a single HPT domain in *Arabidopsis thaliana*. *FEBS Lett.* 437, 11–14 (1998).
21. Suzuki, T., Imamura, A., Ueguchi, C. & Mizuno, T. Histidine-containing phosphotransfer (HPT) signal transducers implicated in His-to-Asp phosphorelay in *Arabidopsis*. *Plant Cell Physiol.* 39, 1258–1268 (1998).
22. Kubo, M. & Kakimoto, T. The *CYTOKININ-HYPERSENSITIVE* genes of *Arabidopsis* negatively regulate the cytokinin-signaling pathway for cell division and chloroplast development. *Plant J.* 23, 385–394 (2000).
23. Becker, D., Kemper, E., Schell, J. & Masterson, R. New binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.* 20, 1195–1197 (1992).
24. Akama, K. et al. Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. *Plant Cell Rep.* 12, 7–11 (1992).
25. McKinney, E. C. et al. Sequence-based identification of T-DNA insertion mutations in *Arabidopsis*: actin mutants *act2-1* and *act4-1*. *Plant J.* 8, 613–622 (1995).
26. Hayashi, H., Crais, L., Lishnow, H., Schell, J. & Walden, R. Activation of a plant gene by T-DNA tagging: auxin-independent growth in vitro. *Science* 258, 1350–1353 (1992).
27. Mumberg, D., Müller, R. & Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156, 119–122 (1995).

Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

## Acknowledgements

M. Higuchi carried out most of the yeast work. We thank H. Saito for yeast strains.

E. Kemper for pGPTV-KAN and K. Torii for comments. Seeds of *abi3-1*, *aux1-7* and *cin2-1* were obtained from ABRC. p415CYC was obtained from ATCC. This study was in part supported by grants from the Ministry of Education, Science and Culture of Japan, and from the Science and Technology Agency to T.K.

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## cdc2 links the *Drosophila* cell cycle and asymmetric division machineries

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Asymmetric cell divisions can be mediated by the preferential segregation of cell-fate determinants into one of two sibling daughters. In *Drosophila* neural progenitors, *Inscuteable*<sup>1–3</sup>, *Partner of Inscuteable*<sup>4,5</sup> and *Bazooka*<sup>6,7</sup> localize as an apical cortical complex at interphase, which directs the apical–basal orientation of the mitotic spindle as well as the basal/cortical localization of the cell-fate determinants *Numb*<sup>8,9</sup> and/or *Prospero*<sup>10,11</sup> during mitosis. Although localization of these proteins shows dependence on the cell cycle, the involvement of cell-cycle components in asymmetric divisions has not been demonstrated. Here we show that neural progenitor asymmetric divisions require the cell-cycle regulator *cdc2*. By attenuating *Drosophila* *cdc2* function without blocking mitosis, normally asymmetric progenitor divisions become defective, failing to correctly localize asymmetric components during mitosis and/or to resolve distinct sibling fates. *cdc2* is not necessary for initiating apical complex formation during interphase; however, maintaining the asymmetric localization of the apical components during mitosis requires *Cdc2/B-type cyclin* complexes. Our findings link *cdc2* with asymmetric divisions, and explain why the asymmetric localization of molecules like *Inscuteable* show cell-cycle dependence.

The embryonic central nervous system (CNS) of *Drosophila* is derived from progenitors called neuroblasts (NBs). NBs undergo repeated asymmetric divisions, budding off a series of ganglion mother cells (GMCs) from their basal/lateral surfaces; GMCs can divide asymmetrically to produce progeny with distinct neuronal fates. Both the NB and GMC asymmetric divisions are mediated, in part, by a protein localization machinery that directs the preferential segregation of *Prospero* (Pros) or *Numb* to the more basally located daughter. Mitosis is driven by activation of the *Cdc2* protein kinase, which, during the first 13 embryonic divisions, depends on dephosphorylation by the product of maternal *string* (*cdc25*)<sup>12,13</sup>. NB divisions occur after depletion of maternal *string* and depend on zygotic *string*. However, NBs, although arrested at G2 of cycle 14, do form in embryos lacking zygotic *string*. In contrast, loss of zygotic *cdc2* does not substantially affect embryonic development, and lethality occurs during postembryonic development<sup>14,15</sup>.

From a mutant screen we identified two lines that exhibited defective localization of Pros and *Inscuteable* (Insc) in NBs. Genetic mapping, complementation and DNA sequencing revealed that the phenotypes associated with both mutants were caused by the same mutation in *cdc2*, resulting in a glutamic acid to glutamine change at amino-acid 51. Embryos homozygous for *cdc2*<sup>ES1Q</sup> show late

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